


Vaccination with human amniotic epithelial cells confer effective protection in a murine model of Colon adenocarcinoma

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As a prophylactic cancer vaccine, human amniotic membrane epithelial cells (hAECs) conferred effective protection in a murine model of colon cancer. The immunized mice mounted strong cross-protective CTL and antibody responses. Tumor burden was significantly reduced in tumor-bearing mice after immunization with hAECs. Placental cancer immunotherapy could be a promising approach for primary prevention of cancer. In spite of being the star of therapeutic strategies for cancer treatment, the results of immunotherapeutic approaches are still far from expectations. In this regard, primary prevention of cancer using prophylactic cancer vaccines has gained considerable attention. The immunologic similarities between cancer development and placentation have helped researchers to unravel molecular mechanisms responsible for carcinogenesis and to take advantage of stem cells from reproductive organs to elicit robust anti-cancer immune responses. Here, we showed that vaccination of mice with human amniotic membrane epithelial cells (hAECs) conferred effective protection against colon cancer and led to expansion of systemic and splenic cytotoxic T cell population and induction of cross-protective cytotoxic responses against tumor cells. Vaccinated mice mounted tumor-specific Th1 responses and produced cross-reactive antibodies against cell surface markers of cancer cells. Tumor burden was also significantly reduced in tumor-bearing mice immunized with hAECs. Our findings pave the way for potential future application of hAECs as an effective prophylactic cancer vaccine.

Introduction

Malignancies are one of the main leading causes of death in human societies. Besides partial effectiveness, toxic side effects particularly on non-cancerous cells have always been an important concern in traditional medical treatments of

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Additional Supporting Information may be found in the online version of this article.

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cancer.¹ As a result, development of new effective therapeutic methods with lesser unwanted side effects is essential. There are numerous and undeniable evidence showing the involvement of immune system in recognition and destruction of cancer cells. In this context, immunotherapeutic approaches have drawn scientist's attention in the past two decades and have been a significant milestone for treatment of different types of cancer.² The most important issue in cancer immunotherapy is the selection of an appropriate target antigen capable of eliciting strong anti-tumor immune responses. In this regard, oncofetal antigens were shown to possess many desirable characteristics to be targeted in cancers.³

The immunologic similarities between cancer and pregnancy were proposed as early as 1884. According to Savory "...before we shall ever be able to answer the question of why or how tumors form ... we must be able to solve the problem of normal growth and development".⁴ Then, Schone reported that vaccination of mice with fetal tissues lead to the rejection of transplanted tumors.⁵ Subsequently, experiments indicated that vaccination of animals with embryonic materials provided surprisingly strong cellular and humoral immunity against implantable tumors, carcinogenic viruses or

What's new?

Cancer stem cells are immunologically similar to embryonic stem cells (ESCs). In particular, both express antigens that are elevated in the presence of cancer. Ethical issues surrounding the use of ESCs have motivated the search for additional cell types with similar stem properties. Our study shows, in a murine model of colon cancer, that placenta-derived human amniotic membrane epithelial cells (hAECs) can elicit a strong anticancer immune response, effectively protecting animals against colon tumor development. In mice with tumors, hAEC treatment lessened tumor burden significantly. The findings suggest that placental cancer immunotherapy is a promising approach for cancer prevention.

chemical carcinogens and supported the idea that anti-tumor immunity arise from the presence of antigens common in fetal tissue and tumor cells.⁶ Similar observations were reported by other investigators.^{7,8}

To unravel molecular mechanisms of such beneficial effects, several investigations have scrupulously identified several candidate antigens common in embryonic tissues and cancer including carcinoembryonic antigen (CEA),⁹ prostate-specific antigen (PSA),¹⁰ survivin,¹¹ Ki-1 (CD30),¹² placental alkaline phosphatase (PLAP),¹³ RCAS1,¹⁴ to list a few.

After a rather long period of scientific gap probably due to technical limitations, the association between pregnancy and cancer was again proposed in a more fascinating form: the cancer stem cell theory.¹⁵ Accordingly, cancer stem cells were shown to possess many similar characters such as antigens and surface markers in common with normal embryonic stem cells (ESCs) which are not expressed in normal tissues and play an important role in metastasis, angiogenesis and chemo resistance.¹⁶ After then, several attempts were made to show protective effects of vaccination with ESCs on cancer development and propagation. Li *et al.* showed that vaccination of mice with human ESC cell line, H9, generated significant cellular and humoral immune responses against CT26 colon carcinoma.¹⁷ Then, Dong *et al.* demonstrated that C57BL/6 mice vaccinated with ESC were cross-protectively immunized against the establishment and expansion of lung cancer.¹⁸ These findings were further supported by additional studies showing that vaccination with ESCs successfully induced activation of anti-tumor immunity.^{19,20} Despite these promising results, ethical problems and the concerns on development of cancer with embryo-derived stem cells have always been the main limitation in using ESCs for clinical application and this may explain why vaccination with fetal materials for tumor immunity has never gone beyond animal models.

Placenta is a unique tissue in the body that exists for a very limited period of pregnancy and provides an exceptional microenvironment capable of controlling invasion of trophoblast cells. Notably, as we and others reported recently, vital parameters of cancer cells are modulated by placental factors and microenvironment²¹ and that placenta expresses such novel markers as PLAC1 (placenta-specific 1) shared by many cancer cell types.^{22,23} It also hosts a collection of cells with stem cell properties.²⁴ More importantly, placental-

derived cells and proteins could effectively hinder the outgrowth of cancer cells in murine models.^{25,26} One such cell type with epithelial origin is human amniotic epithelial cells (hAECs). Similarity of hAECs to embryonic stem cells has been demonstrated by earlier reports.²⁷ Based on the fact that many human cancers are originated from epithelial layers and that hAECs conceptually do not have aforesaid concerns associated with the application of human ESCs due to lack of telomerase activity,²⁴ we were about to examine whether immunization with hAECs could potentially stimulate anti-tumor immune responses and prevent cancer development in a murine model of colon adenocarcinoma.

Materials and Methods**Animals and cell line**

Female 6–8 weeks BALB/c mice, two month old White New Zealand rabbits, BALB/c adenocarcinoma cell lines, CT26 (colon), Renca (kidney) and 4T1 (breast) and C57BL/6 melanoma cell line, B16F10, were obtained from the Pasteur Institute of Iran (Iran, Tehran). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics and incubated in a CO₂ incubator, 37°C. Animals were kept in a standard condition with a 12-hr light–dark cycle and fed *ad libitum*. The animal experiments were carried out using a protocol approved by the Animal Care Committee of Shahid Beheshti University of Medical Sciences and Avicenna Research Institute (Iran, Tehran).

Reagents and antibodies

List of reagents and antibodies used in our study has been provided in Supporting Information.

Multi-lineage differentiation of hAECs

Multi-lineage differentiation potential of hAECs was examined according to the protocols we published recently.^{28–30} Detailed information on osteogenic, chondrogenic and adipogenic differentiation of hAECs has been provided in Supporting Information.

Isolation and immunophenotyping of amniotic epithelial cells

All procedures conducted on human materials were approved by the ethical committee of Avicenna Research Institute and Shahid Beheshti University of Medical Sciences and all

participants signed a written consent form before enrolment to our study. hAECs were obtained from uncomplicated term pregnancies delivered by elective cesarean from 4 healthy women aged 22 to 32 years and characterized using flow cytometry in accordance with the protocols we described previously.³¹ Expression of cytokeratin, CD9, CD10, CD29, CD73, CD34, CD38, CD44, CD105, CD133, HLA-I, HLA DR, HLA-G, SSEA-4, STRO-1 and OCT-4 was assessed by flow cytometry.

Immunization and tumor challenge

Effectiveness of hAECs immunization on induction of anti-tumor responses was investigated in two independent preventive and therapeutic approaches. To study the preventive effect of immunization, 48 female BALB/c mice were randomly divided into three groups of $n = 16$: hAECs vaccine group, CT26 vaccine group (positive control group) and a PBS control group. First and second groups of mice were immunized subcutaneously with 1×10^6 live hAECs and 5×10^5 live 100 Gy gamma-irradiated CT26 tumor cells in 100 μ l PBS, respectively, three times at one week interval. In parallel, control group received 100 μ l PBS subcutaneously. One week after the third vaccination, eight mice from each group were sacrificed and evaluated for their anti-tumor responses, while another eight mice were challenged subcutaneously with 5×10^5 live CT26 cells. To examine potential protective effect of hAECs vaccination against other murine-derived cancer cells, hAECs-vaccinated BALB/c ($n = 10$) and C57BL/6 mice ($n = 10$) were challenged as above with 1×10^5 live 4T1 and B16F10 cells, respectively, and tumor growth and survival were monitored and compared to the control group which received PBS. In the therapeutic approach, the treatment group ($n = 8$) received three hAECs immunizations (with one week interval) eight days after inoculation of CT26, when the touchable tumor masses had developed. Likewise, the control group ($n = 8$) received subcutaneously three PBS injections eight days after CT26 inoculation.

Evaluation of tumor size

Tumor growth was monitored every 3 days using digital calipers to measure length (a, mm) in the direction of the imaginary longitude and the width in the direction of the latitude (b, mm). The tumor area ($a \times b$, mm²) was then calculated. Moreover, mice were followed for their general health symptoms: behavior, feeding and body weight. The mice were euthanized when one dimension of a tumor reached 15 mm or the tumor area of >225 mm² was observed. The primary tumor was also removed and weighed after the mice were sacrificed. According to the Animal Care Committee guidelines and to avoid pain and suffering, survival experiments were not performed on tumor bearing mice.

Evaluation of T cell frequency

One week after the third vaccination, peripheral blood and splenocytes were obtained from mice and the percentage of CD3⁺CD4⁺/CD8⁺ T cells was determined by flow cytometry.

For peripheral blood immunophenotyping, 100 μ l Sodium Citrate-anticoagulated blood was mixed and incubated in the dark with 20 μ l of each monoclonal antibody at 4°C for 20 min. Red blood cells were then lysed by RBC lysis kit according to the manufacturer's recommendation and percentage of positive cell was determined by flow cytometry. In parallel, spleens were excised under sterile condition, splenocytes were separated by perfusion and grinding of spleens and mononuclear cells (MNCs) were isolated by Ficoll density gradient before immunophenotyping. Viability of the isolated cells was tested by trypan blue exclusion test and was always shown to be $>95\%$. One million cells from each spleen were then stained as above.

Measurement of cytotoxic T lymphocyte responses

To evaluate cross-reactive cytotoxic T lymphocyte (CTL) responses against tumor cells after vaccination with hAECs, lactate dehydrogenase (LDH) assay was employed. Briefly, one week after the third immunization, spleen MNCs were isolated as above and washed twice with RPMI-1640. Splenocytes, as effector cells, were seeded into the round-bottom 96-well culture plates in 50 μ l assay medium (RPMI + 1% bovine serum albumin (BSA)) at different numbers (1.25, 2.5, 5×10^5 cells). After a period of 12 hr, target cells (CT26 or hAECs) were added (1×10^4 cells/well) to the wells in triplicate in 70 μ l RPMI-1640 to achieve target/effector ratios of 1:50, 1:25 and 1:12.5, respectively; and plates were then incubated for 6 hr in a 37°C, 5% CO₂ incubator. The target and effector cells cultured alone served as spontaneous LDH release control (low controls). For the maximum LDH release (high control), 2% Triton X-100 was added to the wells containing target cells. The percentage of cytotoxicity was determined based on the following formula:

$$\text{Cytotoxicity (\%)} = \frac{[(\text{effector target cell mix} - \text{effector cell control}) - \text{low control}]/(\text{high control} - \text{low control}) \times 100.}$$

Cytokine measurement

One week after the third immunization, splenocytes were obtained as described above and were co-cultured (1×10^6) with 1×10^5 mitomycin-inactivated target cells in 24-well culture plates in a final volume of 1 ml complete medium (-RPMI-1640 supplemented with 12% FBS) per well. After 48 hr, supernatant was collected and tested for the levels of IL-10, IL-4 and IFN- γ by ELISA sets as per manufacturer's instructions. The optical densities at 570 and 450 nm (as a reference wavelength) were recorded by an ELISA reader (Anthos, Austria) and concentration of each cytokine was determined using the corresponding standard curve. The minimal detection limits for IL-10, IFN- γ and IL-4 were 31.3, 31.3 and 7.3 pg/ml, respectively.

Analysis of cross-reactive antibody responses by immunofluorescent staining and flow cytometry

In order to investigate cross-reactive antibody responses against CT26 cells in mice receiving hAECs, blood sample

was collected via cardiac puncture one week after the third immunization and serum was separated. CT26 and hAECs cells (as positive control) were harvested, cytospinned and fixed with ice cold acetone for 2 min. Cells were then washed twice with PBS + 0.5% BSA each for 5 min. The cells were incubated for 30 min at room temperature (RT) with PBS containing 5% sheep serum and 2.5% BSA to block non-specific reactions followed by three washing steps with PBS. Afterward, the cells were incubated with 1:200 dilution of hyper immune mouse sera for 90 min. Negative reagent control slides received non-immune mouse serum with the same dilution. After being washed with PBS, cells were incubated with FITC-conjugated sheep anti-mouse Ig (diluted 1:50) at RT for 45 min and washed with PBS as above. The nuclei were stained with DAPI (0.5 µg/ml). Finally, the cells were examined under fluorescence microscope (Olympus BX51, Japan) equipped with DP71 CCD camera. As control, reactivity of sera from hAECs-immunized BALB/c mice with 4T1 and Renca and that of hAECs-immunized C57BL/6 sera with B16F10 cells were tested as described above. To examine whether or not induction of cross-reactive antibody responses is species-specific, New Zealand white rabbits were immunized subcutaneously with live hAECs four times with two week interval and reactivity of hyper immune sera was then tested on hAECs and CT26 cells as above. Indeed, reactivity of rabbit polyclonal anti-hAECs antibody was also tested by flow cytometry. To this end, hAECs and CT26 cells were washed with PBS containing 1% FBS (PBS-FBS) and incubated with 1:200 dilution of hAECs-immunized rabbit sera for 30 min on ice. After washing with PBS-FBS, cells were incubated with FITC-labeled sheep anti-rabbit Ig for 20 min and analyzed by flow cytometry as above. Cells incubated with the same dilution of pre-immune rabbit serum were used as control.

Statistical Analysis

The numerical values of the results were presented as mean ± SEM. Comparisons between groups were done by Kruskal Wallis and Mann-whitney. *P* values <0.05 were considered to be significant. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, <http://www.graphpad.com>) software.

Results

hAECs expressed markers of mesenchymal and embryonic origin

About 80–130 × 10⁶ hAECs were isolated from each placenta unit with high purity as judged by assessment of cytokeratin expression (≥ 90%). Trypan blue dye exclusion test revealed a viability of >95%. These cells appeared as flat round cells with abundant cytoplasm and high cytoplasm: nuclear ratio. Immunophenotyping of hAECs was performed using flow cytometry. Accordingly, hAECs were positive for cytokeratin, CD9, CD10, CD29, CD73, CD105, HLA-I, HLA-G, STRO-1,

SSEA-4 and OCT-4 while they were negative for HLA-II, CD34, CD38, CD44 and CD133 (Fig.1a).

hAECs showed multi-lineage differentiation potential

Nodule-like structures with calcium deposits were formed with a strong positive staining for Alizarin red (Fig.1bb). Moreover, Alcian blue staining showed abundant accumulation of proteoglycan in differentiated cells into chondrocytes (Fig.1bd). In contrast to differentiated cells, undifferentiated cells were negative in both staining procedures (Fig.1ba and bc). Despite several attempts for adipogenic differentiation of hAECs, they displayed the absence or very faint staining for lipids (Fig.1bf).

Vaccination with hAECs conferred protection in a murine model of colon cancer

BALB/c mice were immunized with irradiated CT26 cells, live hAECs, or PBS and challenged with living CT26 cells thereafter (Fig. 2a). The rate of tumor growth in terms of size and weight was then followed closely until 35 days post tumor inoculation. Remarkably, vaccination with both CT26 and hAECs resulted in complete inhibition of tumor development in all vaccinated mice compared to the control group (****p* < 0.001) (Fig. 2b and 2c). However, in control mice receiving PBS, tumors were developed and increased in sized steadily (Fig. 2d). We also tested the potential of hAECs vaccination in protecting mice against breast (4T1) and melanoma (B16F10) cancer cells. We found that hAECs vaccination did not confer protection against 4T1 cells in terms of survival rate (Fig. 2e) or tumor growth (Fig. 2f). However, Mice vaccinated with hAECs were significantly protected against B16F10 challenge (Fig. 2g). Indeed, hAECs vaccination significantly delayed tumor development and reduced tumor weight in vaccinated C57BL/6 mice (Fig. 2h) (*p* < 0.0001).

Vaccination with hAECs led to expansion of systemic and splenic cytotoxic T cell population

To determine whether hAECs immunization resulted in expansion of CD4⁺ and CD8⁺ T lymphocytes, we measured their frequency in peripheral blood (Fig. 3a and 3b) and spleen (Fig. 3a and 3c) of vaccinated mice. Our data notably demonstrated that the percentages of peripheral blood and splenic T cells (CD3⁺) in both hAECs and CT26 vaccine groups were significantly higher compared to those in the control group (*p*<0.01–0.001). Of note, hAECs-vaccinated mice had higher frequency of peripheral and splenic CD4⁺ T (*p*< 0.01–0.001) and CD8⁺ cells (*p*< 0.05–0.001) compared to the control group. CT26-immunized mice showed the similar trend of T cell expansion, although the level of splenic T cell expansion was even higher in this group compared to hAECs-immunized mice (*p*<0.01).

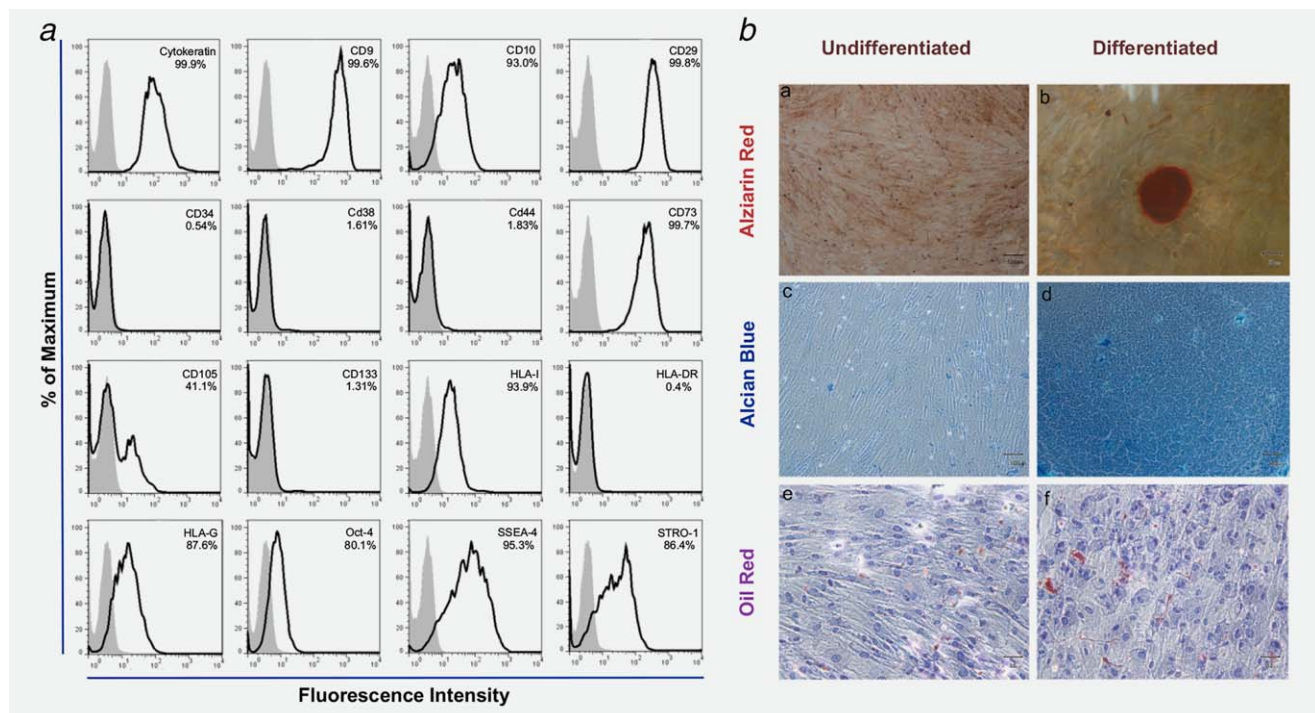


Figure 1. hAECs exhibit multilineage differentiation potential and a mixed mesenchymal and embryonic phenotype. (a) Expression of markers associated with mesenchymal and embryonic origin was assessed by flow cytometry. The results are representative of three independent experiments. In each graph, open and filled histograms represent test and isotype-matched control antibodies, respectively. (b) Freshly-isolated human amniotic membrane epithelial cells were induced to differentiate toward osteocytes, chondrocytes and adipocyte and degree of differentiation was verified by Alizarin red, Alcian blue and Oil red stainings, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

Vaccination with hAECs-induced tumor-specific, cross-protective cellular immunity against CT26 cells

The data depicted in Figure 3d indicate that splenocytes from both CT26 and hAECs vaccine groups showed strong CTL activity against hAECs at all target: effector (T:E) ratios ($p < 0.01$ – $p < 0.001$). In parallel, splenocytes from hAECs-vaccinated mice exhibited strong CTL activity against CT26 cells at all T:E ratios ($p < 0.001$), whereas those from control mice did not show cytotoxicity. As expected, mice receiving CT26 cells as vaccine showed very strong CTL activity against CT26 cells which were significantly higher compared to both control and hAECs vaccine group ($p < 0.05$ – $p < 0.001$) (Fig. 3e).

Immunization with hAECs significantly increased IFN- γ production in response to CT26 challenge

In order to further investigate the immune mechanism underlying anti-tumor immunity in hAECs-vaccinated mice, the levels of interleukin (IL)-4 (Fig. 3f), IFN- γ (Fig. 3g) and IL-10 (Fig. 3h) in culture supernatant of splenocytes stimulated with either hAECs or CT26 cells were measured. The results showed that immunization with hAECs cells induced a mixed cytokine profile in splenic cells. The level of IFN- γ increased significantly after stimulation with either hAECs or CT26 ($p < 0.01$). The same trend was also observed in mice

vaccinated with CT26 ($p < 0.01$ – 0.001). Mice vaccinated with CT26 produced even more IFN- γ compared to those vaccinated with hAECs ($p < 0.01$). Although hAEC- or CT26-vaccinated mice produced higher amounts of IL-4 after stimulation with immunizing cells compared to the PBS group ($p < 0.05$ – 0.01), stimulation of splenocytes with hAECs or CT26 in hAEC-vaccinated mice resulted in a significantly lower IL-4 production compared to the cells remained unstimulated ($p < 0.05$). In parallel, spleen cells of hAECs- or CT26-vaccinated mice increased IL-10 production after stimulation with either hAECs or CT26 ($p < 0.01$ – 0.001). Non-stimulated splenocytes of hAECs- or CT26-vaccinated mice also showed higher tendency to produce IL-4, IL-10 and IFN- γ compared to those from PBS group ($p < 0.05$ – 0.001). hAECs or CT26 cells when cultured alone produced no detectable levels of cytokines (data not shown).

Cross-reactive antibodies were generated against CT26 cells after vaccination with hAECs

Our results clearly showed that sera from the control group mice reacted neither with hAECs nor CT26. Sera from hAECs- and CT26-vaccinated mice sharply cross-reacted with CT26, and hAECs cells, respectively. Interestingly, sera from hAECs-vaccinated mice recognized antigens in CT26 cells which were predominantly located at cell surface. As

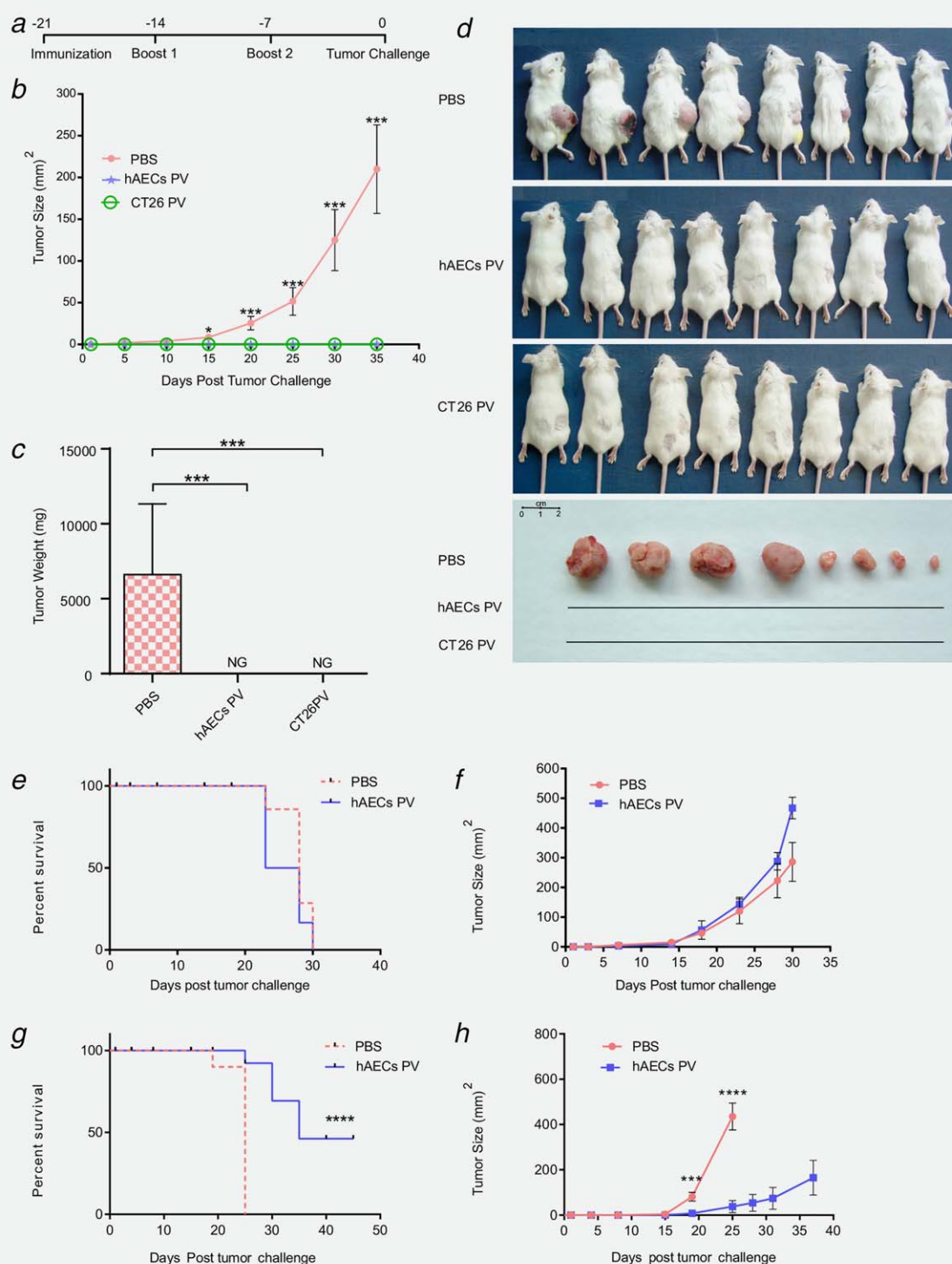


Figure 2. Prophylactic vaccination with hAECs confers protection against murine colon carcinoma. (a) Vaccination Scheme: PBS, 1×10^6 hAECs, or 5×10^5 irradiated murine colon cancer cell line, CT26, was injected subcutaneously into BALB/c mice ($n = 8$) in phosphate buffered saline three times at one week interval. Mice were challenged seven days after the last vaccination with 5×10^5 wild type CT26 cells. (b) Tumor volumes were monitored regularly and calculated by measuring tumor dimensions with digital calipers. (c) Final tumor weights were measured on the excised tumors at the end of the experiment. (d) Immunization with hAECs or inactivated CT26 cells inhibited tumor growth. Indeed, hAECs-vaccinated BALB/c ($n = 10$) and C57BL/6 ($n = 10$) mice were challenged with 1×10^5 4T1 and B16F10 cells, respectively, as above. hAECs vaccination did not confer protection against 4T1 cells in terms of survival rate (e) or tumor growth (f). However, Mice vaccinated with hAECs were significantly protected against B16F10 challenge (g). hAECs vaccination significantly delayed tumor development and reduced tumor weight in vaccinated C57BL/6 mice (h) ($p < 0.0001$). Error bars denote mean \pm SEM. Asterisks (*) indicate statistical significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). hAECs: human amniotic epithelial cells, PBS: Phosphate-buffered saline, NG: No growth. [Color figure can be viewed at wileyonlinelibrary.com]

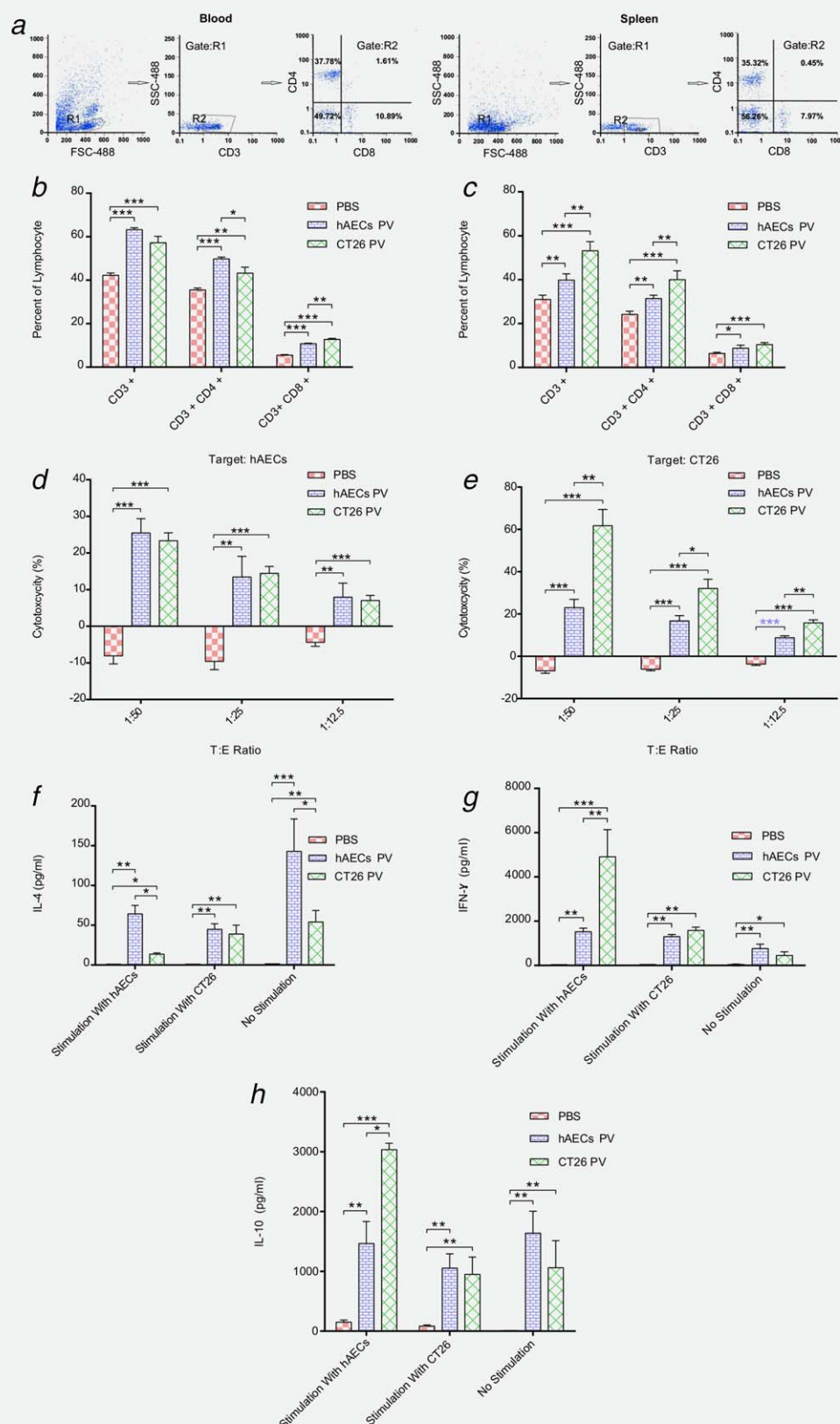


Figure 3. Vaccination with hAECs augments cross-reactive cellular immunity and CTL responses against murine colon carcinoma. Gating strategy of blood and spleen immune cells has been shown (a). Prophylactic vaccination with hAECs induced a significant proliferation of CD4⁺ and CD8⁺T lymphocytes in the peripheral blood (b) and spleen (c) of vaccinated mice compared to the control group ($n = 8$). The potential of hAECs vaccination to induce CTL responses was tested by LDH cytotoxicity assay using splenocyte of vaccinated mice as effector cells and hAECs (d) or CT26 cells (e) as targets. The percentage of specific lysis was measured at different target: effector (T:E) ratios. Vaccination with hAECs cells induced cross-reactive CTL responses against CT26 cells at all T:E ratios tested and *vice versa*. One week after the last prophylactic vaccination, the levels of IL-4 (f), IFN- γ (g) and IL-10 (h) were measured in culture supernatants of splenocytes stimulated with either hAECs or mitomycin-inactivated CT26 using ELISA. In some wells, cells remained unstimulated. Error bars denote mean \pm SEM. Asterisks (*) indicate statistical significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). hAECs: human amniotic epithelial cells, PBS: Phosphate-buffered saline, PV: Prophylactic vaccine. [Color figure can be viewed at wileyonlinelibrary.com]

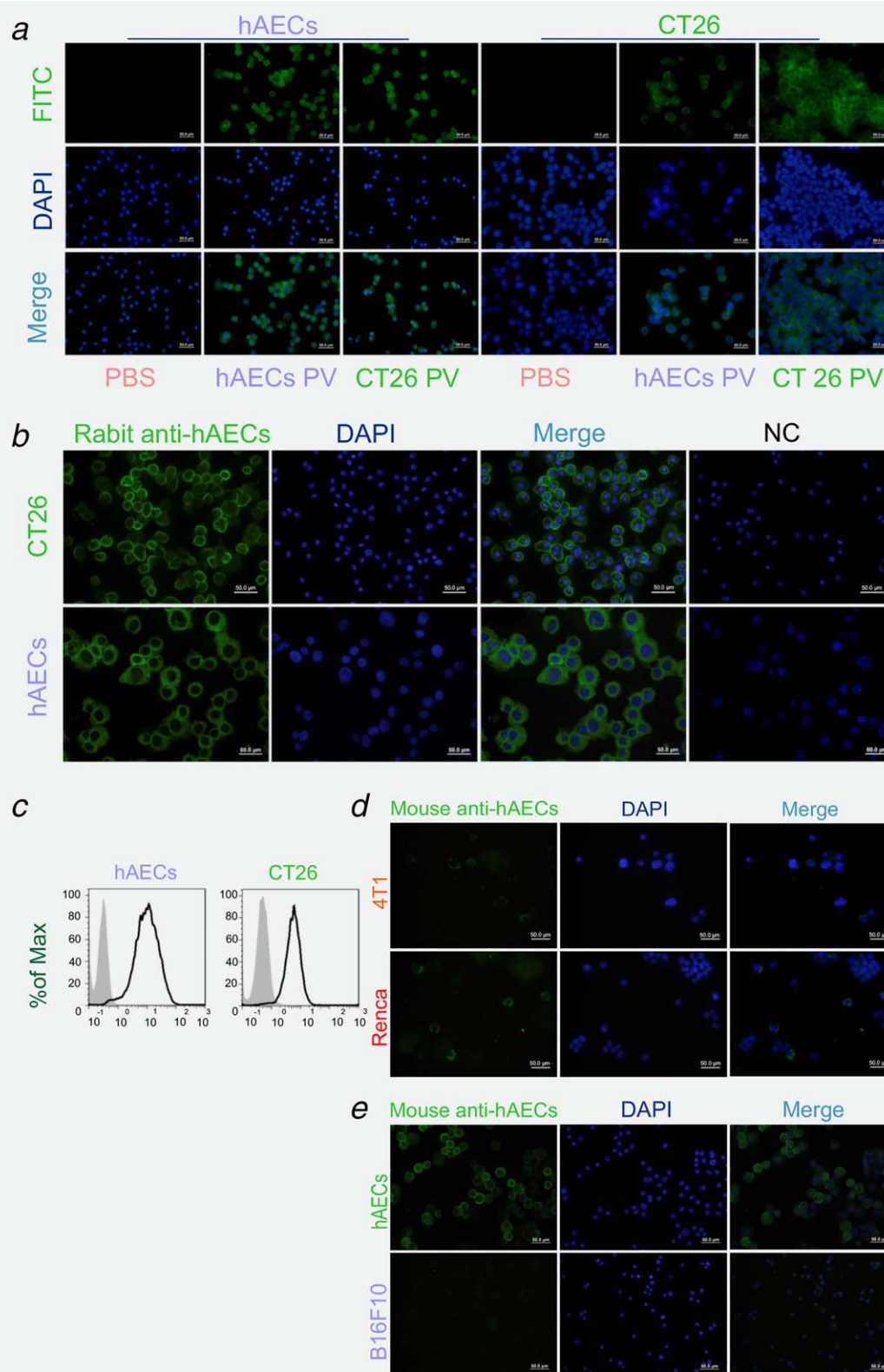


Figure 4. Immunization with hAECs generate cross-reactive antibodies against CT26 cells. (a) Mice ($n = 8$) were immunized with either hAECs or CT26 cells and immune reactivity of sera from each group was tested against both CT26 and hAECs. hAECs-induced antibodies strongly recognized cross-reactive molecules in CT26 cells which were mostly localized to cell membrane. Similar pattern was also observed when sera of CT26-vaccinated mice were applied on hAECs cells. As positive control, sera from each vaccine group were separately tested against immunizing cells and the results were shown always to be positive. (b) Sera from rabbits immunized with hAECs also showed strong reactivity with CT26 and immunizing cells with cell surface staining pattern. (c) Flow cytometric analysis of sera from hAECs-immunized rabbits also showed that antibodies directed against hAECs could efficiently recognize native cell surface antigen(s) in CT26 cells. (d) Immunofluorescent staining of 4T1 and Renca cells with sera from hAECs-immunized mice showed weak reactivity in a small proportion of cells. (e) No cross-reactive antibodies were found against B16F10 cells in sera of hAECs-vaccinated C56BL/6 mice, while a strong immunoreactivity, predominantly localized to the cell surface markers, was observed when sera from hAECs hyper immune sera of C56BL/6 mice were tested against hAECs cells as positive control. Cell nuclei were stained with DAPI. NC: Negative control.

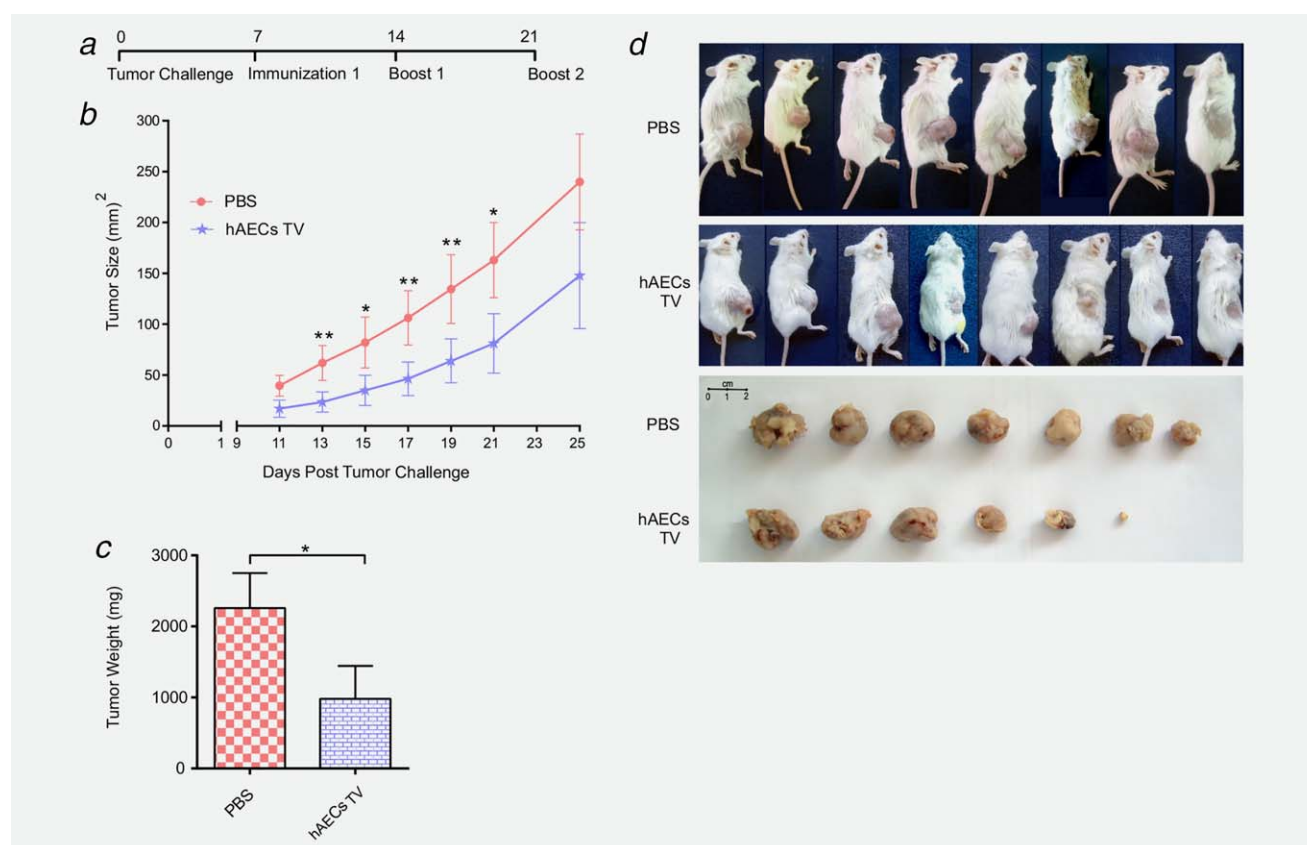


Figure 5. Treatment with hAECs reduces tumor burden in murine colon carcinoma. (a) Immunization schedule: Eight days after inoculation with CT26, when palpable tumor mass was developed, treatment group ($n = 8$) received three hAECs immunization subcutaneously at one week interval, while control group ($n = 8$) received PBS. Tumor volume (b) was monitored regularly over the experimental period. (c) Final tumor weight of the excised tumors was measured at the end of the experiment. (d) Immunization of tumor-bearing mice significantly reduced tumor size. Error bars denote mean \pm SEM. Asterisks (*) indicate statistical significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). hAECs: human amniotic epithelial cells, PBS: Phosphate-buffered saline, TV: Therapeutic vaccine. [Color figure can be viewed at wileyonlinelibrary.com]

positive control, sera from each vaccine group were separately tested against immunizing cells and the results were shown always to be positive (Fig. 4a). In line with the results obtained from hAECs-immunized mice, sera from rabbits immunized with hAECs showed strong positive reaction with both immunizing cells and also with CT26 cell line which was predominantly localized to cell membrane (Fig. 4b). These results were further validated by flow cytometric analysis of sera from hAECs-immunized rabbits. The results clearly showed that antibodies directed against hAECs could efficiently recognize native cell surface antigen(s) in CT26 cells (Fig. 4c). To rule out the possibility that production of cross-reactive antibodies against CT26 after immunization of mice with hAECs is not due to the xenogeneic response, or resulted from probable FCS contamination during immunization, hyper immune BALB/c mice sera against hAECs were also tested for reactivity with other mouse-derived cancer cell lines, 4T1 and Renca. As depicted in Figure 4d, most of 4T1 and Renca cells failed to react with anti-hAECs sera. However, a small proportion of cells reacted with anti-hAECs sera, although the signal was comparatively weaker than those obtained with CT26 cells. In parallel, reactivity of hAECs

hyper immune C56BL/6 mice with B16F10 cells was also tested. The results showed no antibody reactivity against B16F10 cells in sera of C56BL/6 mice immunized with hAECs, while a strong immunoreactivity, predominantly localized to the cell surface markers, was observed when sera from hAECs hyperimmune sera of C56BL/6 mice were tested against hAECs cells as positive control (Fig. 4e).

hAECs immunization exerted therapeutic effect after tumor establishment

To determine whether treatment with hAECs could suppress tumor progression, tumor-bearing mice received three series of hAECs immunization one week after inoculation of CT26 cells (Fig. 5a) and tumor growth was monitored every 3 days (Fig. 5b). Of note, tumor growth was significantly retarded between 13–21 days post tumor inoculation in mice receiving hAECs compared to the control group. After sacrificing the mice, tumors were removed and average tumor weight was measured. Likewise, tumor weight was significantly lower in mice receiving hAECs compared to the PBS-treated control mice ($p < 0.05$). (Fig. 5c and 5d).

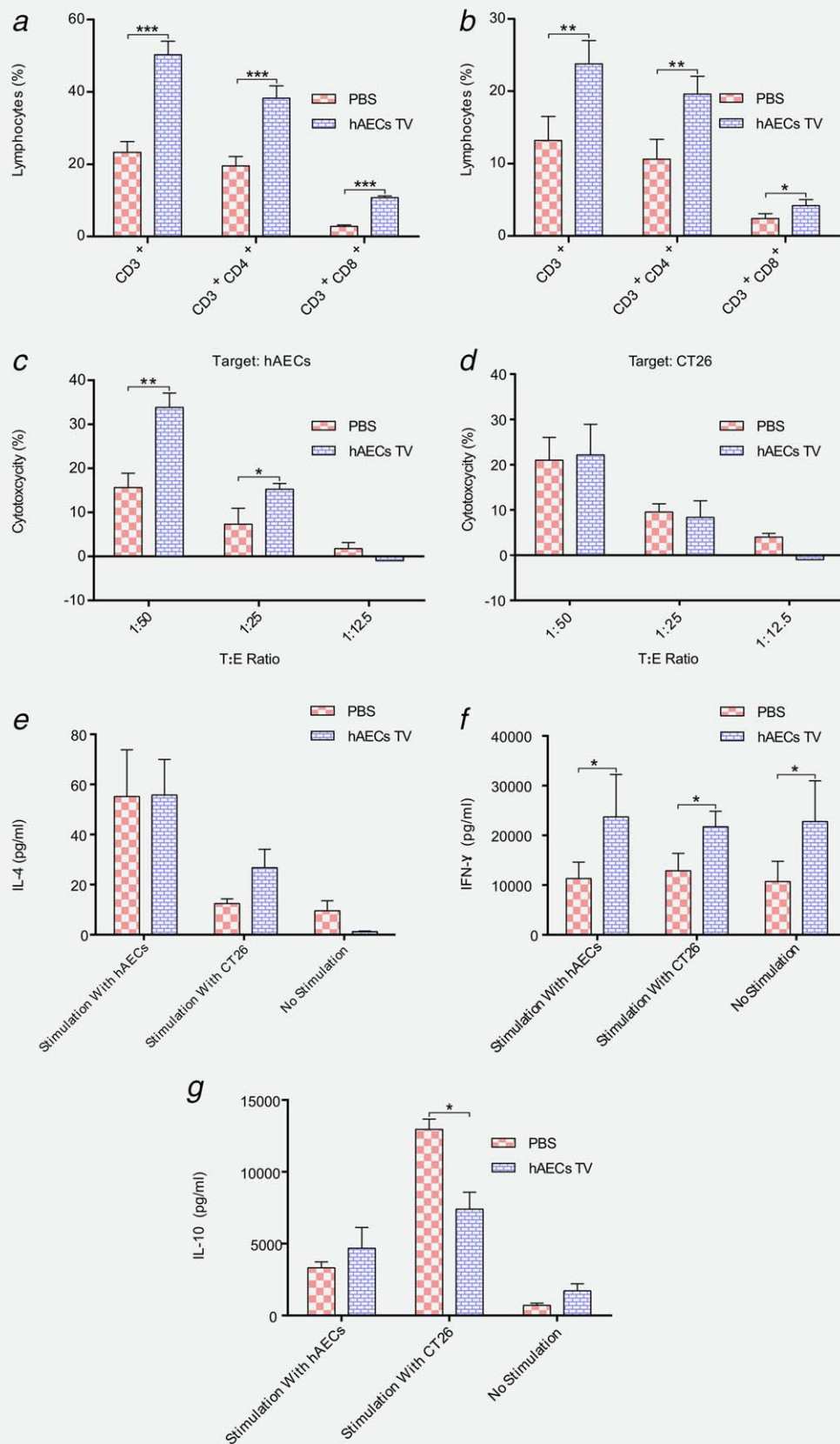


Figure 6. Treatment with hAECs increases Th1 immunity and CD8⁺ T cell frequency but not CTL activity. Immunization of tumor-bearing mice with hAECs induced a significantly higher proliferation of T and CD8⁺ T lymphocytes in the peripheral blood (a) and spleen (b) of mice compared to the control group ($n = 8$). CTL activity of splenocytes from hAECs-treated mice was measured by LDH cytotoxicity assay against hAECs (c) and CT26 cells (d). The percentage of specific lyses was measured for different targets: effectors (T:E) ratios. Immunization with hAECs induced significant specific CTL activity against the same cells at 1:50 and 1:25 T:E ratios, while exerted no significant CTL activity against CT26 cells. One week after the last prophylactic vaccination, the levels of IL-4 (e), IFN-γ (f) and IL-10 (g) was measured in culture supernatants of splenocytes stimulated with either hAECs or mitomycin-inactivated CT26 using ELISA. Treatment with hAECs caused a significant increase of IFN-γ producing cells, while decreased IL-10 production level in response to CT26 stimulation. Error bars denote mean \pm SEM. Asterisks (*) indicate statistical significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). hAECs: human amniotic epithelial cells, PBS: Phosphate-buffered saline, TV: Therapeutic vaccine. [Color figure can be viewed at wileyonlinelibrary.com]

Treatment with hAECs augmented the expansion of peripheral blood and splenic CD8⁺ T cells

The percentages of CD3⁺ T cells in general and CD8⁺ T lymphocytes in particular in peripheral blood (Fig. 6a) and spleen (Fig. 6b) of mice treated with hAECs were significantly superior to that of the control group ($p < 0.05$ – $p < 0.001$). The percentage of CD4⁺ T cells in peripheral blood and spleen of vaccinated mice was also increased significantly compared to the control group ($p < 0.01$ – $p < 0.001$).

Treatment with hAECs did not induce significant tumor-specific CTL responses

As expected, injection of hAECs to tumor-bearing mice induced a significant specific cellular immunity against hAEC target cells compared to the control group (Fig. 6c). However, this treatment did not induce a significant increase in CTL responses against CT26 cells compared to the control group (Fig. 6d). In contrast to the mice receiving PBS as vaccine (Fig. 3c and 3d), splenocytes from PBS-treated tumor-bearing mice group exhibited specific lysis against hAECs and CT26 target cells (Fig. 6c and 6d).

Treatment with hAECs potentiated Th1 responses

Our results showed that treatment with hAECs caused a significant increase in IFN- γ -producing cells in the spleen of tumor-bearing mice ($p < 0.05$), while such treatment resulted in a decreased IL-10 production level compared to the PBS group. Production of IL-4 was not affected by hAECs treatment (Fig. 6e–6g).

Discussion

One of the most promising, yet challenging, modalities for cancer treatment is to mobilize the immune system to combat against malignancy. In this context, targeting tumors by different arms of the immune system has been the focus of many basic and clinical researches for over a century.²

Very earlier than introduction of conventional cancer immunotherapeutic strategies employing monoclonal antibodies and adaptive cellular therapy, similarities between cancer and embryonic tissues were noticed. Several studies clearly corroborated that cancer and embryonic cells have many antigens in common so that immunization of animals with embryonic tissues could induce effective anti-tumor responses.³² A large proportion of vaccine trials conducted so far for cancer immunotherapy have been based on such embryonic antigens as carcinoembryonic antigen,³³ cancer/testes antigens³³ and α -fetoprotein,³⁴ to list a few. However, it is very unlikely that monovalent cancer vaccines employing a single antigen could enlist effective anti-tumor immunity mainly because of the fast emergence of diverse immune escape mechanisms by the tumor cells.^{35,36} Interestingly, extensive similarities in markers expressed by embryonic and cancer stem cells prompted the researchers to take advantage of cross-protective immune responses induced by ESCs

immunization for cancer treatment. Li *et al.* showed the potential of human ESCs to efficiently immunize against murine colon cancer.¹⁷ These findings were further supported by the experiments from other investigators showing that vaccination with human ESCs lead to robust immune activation against malignant colon, lung and ovarian cancers and effective suppression of proliferation of cancer cells.^{18–20} However, due to the ethical problems and fear of creating teratoma because of the telomerase activity in ESCs, their utility in cancer immunotherapy is subjected to a serious concern.

Here, we examined potential of hAECs for being applied as preventive and therapeutic cancer vaccine in a mouse model of colon adenocarcinoma. We first investigated stem cell marker signature of hAECs and showed in line with what reported earlier^{31,37} that they express well-defined human mesenchymal stem cell markers (CD9, CD10, CD29, CD73, CD105), as well as the embryonic stem-cell markers, Oct-4, SSEA-4 and STRO-1. Their stemness was further confirmed by osteogenic and chondrogenic differentiation potential. Adipogenic potential of isolated hAECs was also tested. In line with Diaz-Prado *et al.*³⁸ hAECs displayed the absence or very faint staining for lipids.

We next showed for the first time that mice immunized with hAECs resisted tumor induction with highly tumorigenic CT26 colon cancer cell line. In fact, none of the mice vaccinated with hAECs developed tumor till the end of Experiment (35 d post-inoculation) despite injection of CT26 cells five times higher as much as the inoculum size required for tumor induction.³⁹ Our results clearly showed that vaccination with pre-inactivated CT26 cells could effectively confer protection against the same tumor. In our study, we observed strong anti-tumor cellular responses after vaccination with either hAECs or CT26 exemplified by a significantly higher frequency of peripheral blood and spleen T and cytotoxic CD8⁺ T cells which play a crucial role in anti-tumor immunity.⁴⁰ Interestingly, assessment of functional CTL activity revealed that immunization with hAECs induced a robust CTL activity against CT26 cells at all T:E ratios tested and *vice versa* implying induction of anti-tumor CTL responses by antigens shared by hAECs and CT26 cells. It is possible that antigens expressed by hAECs cells are cross-presented by mouse MHC molecules and activate CD4⁺ and CD8⁺ cells. To determine whether such cross-protection capability of hAECs is confined to the colon adenocarcinoma, we also tested anti-tumor vaccination potential of hAECs in breast cancer and melanoma models. We observed that hAECs vaccination had no obvious effect in inhibiting 4T1 breast cancer cell growth, while significantly increased survival and prohibited tumor growth in a mice model of melanoma. These results clearly indicated that anti-tumor vaccination potential of hAECs is cancer type-dependent. It remains to be determined which mechanisms are responsible for such differential protective effect of hAECs against cancers from histologically different origins.

In parallel, sera from hAECs-vaccinated mice and rabbits sharply recognized yet uncharacterized common surface markers in CT26 and hAECs. In support of our findings, several other investigators reported cross-reactive antibodies directed against ovary (NuTu-19 Cells)⁴¹ and colon (CT26 Cells)¹⁷ cancer cells after immunization of mice with embryonic stem cells. In contrast, we found minimal or no cross-reactive antibody responses in hAECs-vaccinated BALB/c or C57BL/6 mice against 4T1 and B16F10 cells, respectively. These results may indicate that hAECs antigens leading to the induction of cross-reactive antibodies are not shared by all cancer cell types.

The nature of such antigen(s) remain to be elucidated, however, oncofetal antigens are most likely to be the target antigens in induction of cross-protective immunity. Nonetheless, epitope spreading after hAECs-induced immune elimination of CT26 cells could also potentially lead to immune responses against antigenic targets in CT26 cells not expressed by hAECs cells.⁴² We do not think that hAECs-induced anti-tumor immunity is due to a non-specific xenogeneic response as iPS cells from human origin (TZ1) was shown to fail to inhibit tumor growth in the same model of murine colon carcinoma.¹⁷ Indeed, our results showed that anti-AEC hyper immune serum exhibit reactivity only with small proportion of other mouse-derived cell lines, 4T1 and Renca, or showed no reactivity at all with B16F10 melanoma cells ruling out the possibility of non-specific xenogeneic responses after hAECs immunization.

Although immune responses against antigens shared by hAECs and CT26 cells seems to be a fascinating explanation for hAECs-triggered resistance to colon cancer development, direct anti-tumor properties of hAECs could be regarded as a further explanation as reported by Kang *et al.*⁴³ Anti-angiogenic and -proliferative potential of hAECs are among further well-characterized anti-cancer capacity of this cell type⁴⁴ which are also employed by amniotic membrane inner cell population, amniotic mesenchymal cells.⁴⁵ To investigate further mechanisms responsible for immunity against tumor establishment after hAECs vaccination, we next measured cytokine levels produced by the peripheral blood and splenic mononuclear cells. Our results showed that akin to mice immunized with CT26, mice received hAECs prophylactic vaccine produced a mixed pattern of cytokines attributed to Th1, Th2 and regulatory T cells. Considering that specific activity of different cytokines differs profoundly in essence and is also affected by the experiment condition, we could not come to a net conclusion that which cytokine profile prevails after vaccination with hAECs. Indeed, mechanisms responsible for immunity against cancers are so complex that no single immunological mechanism could certainly be ascribed to tumor rejection. It should be noted that considerable amounts of cytokines were produced without stimulation by splenocytes of hAECs- or CT26-vaccinated mice *in vitro*. This pattern could be attributed to the continuation of

immunological responses initiated after vaccination as such pattern was not observed in the control mice.

At present, it is not clear for us how long protective immunity against cancer after hAECs vaccination lasts and additional follow up studies are needed to be performed, but such immunity would most likely wane over time⁴⁶ necessitating regular strengthening of memory responses.

Besides effectiveness of hAECs cells as a prophylactic cancer vaccine, we were also interested to know to what extent immunization with this cell type could be effective against a pre-established tumor. Our results showed that when injected to the tumor-bearing mice, hAECs considerably reduced tumor burden. Although, treatment of tumor-bearing mice with hAECs resulted in an increased frequency of T and CD8⁺ T cells in blood and spleen, not surprisingly CTL activity against cancer cells was not affected. This finding is in line with previous reports indicating that tumor progression in experimental models and in humans is associated with a functional impairment in cancer antigen-specific CD8⁺ T cells.⁴⁷ Lyman *et al.* clearly showed that cancer antigen-specific CTLs did not mount an effector function in case of increasing tumor burden and tumor antigen availability.⁴⁸ In another study, despite presence of CD8⁺ T cells displaying an antigen-experienced phenotype in regional draining lymph nodes, these cells were typically deficient in one or more effector functions; a phenomenon termed "split energy".⁴⁹ Interestingly, CTL activity against hAECs was increased in hAECs-treated tumor-bearing mice indicating that induction of CTL tolerance to tumor antigens in the course of a cancer is fundamentally cancer stage-dependent. Nonetheless, increased IFN- γ and decreased IL-10 production by spleen cells of hAECs-treated mice in response to CT26 stimulation could be viewed as one possible mechanism responsible for induction of Th1 immunity and reduction of tumor load, although there might be other mechanisms and factors involved.

One main question that needs to be addressed is why hAECs-vaccination confers protection against colon cancer development while the same cells are only partially effective in reduction of tumor burden when the cancer is established? It can be postulated that pre-existing immunity against cancer cells might serve as a safe guard hindering proliferation of cancer cells by induction of cell cycle arrest and apoptosis. In this context, cancer cells do not find an opportunity to propagate and organize into a tumor. In contrast, the ability of pre-established tumor to foster a tolerant microenvironment and activation of a plethora of immunosuppressive mechanisms⁴⁷ prevents hAECs-induced anti-tumor responses to be fully effective. In fact, quality of interaction between tumor cells and the immune system is markedly a function of time elapsed after initiation of carcinogenesis. In the very early stages, most of the cancer cells are eliminated by well-defined immune surveillance mechanisms. However, when tumor is established, growing cancer cells enhance their self-protection via multiple immune evasion mechanisms and anti-cancer immunity induced by therapeutic vaccination is unable to exert full spectrum of anti-oncogenic activity.⁵⁰ In

support of this notion, Dong *et al.* demonstrated that administration of ESCs five days after inoculation of lung carcinoma cells was significantly less effective in tumor size reduction compared to when mice were immunized with these cells two days after tumor challenge.¹⁸

In conclusion, the results of our study clearly demonstrated that hAECs possess the potential for being used as an effective prophylactic vaccine for immune prevention of colon cancer most probably due to the shared antigenic determinants. The nature of protective antigens responsible

for tumor rejection, capacity of hAECs to confer protection against other cancer types and assessment of cancer preventive efficacy of purified candidate hAECs antigens warrants further investigations before hAECs-based prophylactic cancer vaccines move into clinical trials.

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